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3 **Influences of swab types and storage temperatures on isolation and molecular detection of**

4 ***Mycoplasma gallisepticum* and *Mycoplasma synoviae***

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Abstract

Routine diagnosis of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) is performed by collecting oropharyngeal swabs, followed by isolation and/or detection by molecular methods. The storage temperature, storage duration and the type of swabs could be critical factors for a successful isolation or molecular detection. The aim of this study was to compare the influence of different types of cotton tipped swabs stored at different temperatures, on detection of MG and MS. To achieve this, a combined use of traditional culture analysis (both agar and broth), with modern molecular detection methods was utilised. Performances of wooden and plastic shaft swabs, both without transport medium, were compared. Successful culture of *M. gallisepticum* was significantly more efficient from plastic swabs when compared to wooden, whereas no difference was seen for re-isolation of *M. synoviae*. Storage at 4 oC compared to room temperature also increased the efficiency of culture detection for both Mycoplasma species. When stored at room temperature, PCR detection limits of both MG and MS were significantly lower for wooden compared to plastic swabs. The qPCR data showed similar detection limits for both swab types when stored at both temperatures. Results suggest that swabs with plastic shaft should be preferred for MG and MS detection by both culture and PCR. While a lower storage temperature (4°C) is optimal for culture recovery, it seems that both temperatures investigated here are adequate for molecular detection and it is the swab type which carries a greater influence.

Keywords: wooden swabs, plastic swabs, temperature, *Mycoplasma gallisepticum*, *Mycoplasma synoviae,* detection

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are important poultry pathogens worldwide, both responsible for substantial economic losses. Oropharyngeal swabs collected from suspected infected flocks are routinely analyzed to confirm the presence of mycoplasmas by culture and/or molecular methodology. Sample storage temperature and the type of swab could influence successful detection (Christensen *et al.*, 1994; Zain and Bradbury, 1995; Zain and Bradbury, 1996; Daley et al., 2006; Ferguson-Noel *et al.*, 2012). The use of a suitable transport media (such as mycoplasma broth or charcoal) has been advised for transportation of samples.

As favorable transportation of samples for culturing may be the most important factor affecting successful detection of mycoplasmas (Drake *et al.*, 2005), it is important to consider field samples normally arrive at the laboratory 1-3 days after sampling. For PCR detection of MG or MS, results can be influenced by various factors, including the amount of DNA recovered, which could depend on type of swabs used, as well as the DNA extraction method (Brownlow *et al*, 2012).

The aim of this study is to compare two types of dry cotton swabs (wooden *versus* plastic shafts) which were stored at two different temperatures. Additionally, we investigated the effect of a longer duration between sample taking and laboratory processing. The influences of these factors on detection of MG and MS by isolation, and conventional and real-time PCR were assessed.

Materials and methods

Mycoplasma strains and culture

Two mycoplasma type strains were used throughout the study: MG PG31 and MS WVU 1853. Both strains were titrated using the viable counts method according to Miles *et al*. (1938) and

2 expressed as colony-forming units (CFU)/ml. Briefly, strains were ten-fold diluted up to 10-7 in
73 mycoplasma broth (MB). Then, 100 µl of each strain dilution were inoculated onto mycoplasma
74 agar (MA) plates, using one plate per dilution. Both broth and agar media were prepared as
75 previously reported (Bradbury, 1977; Zain and Bradbury, 1995). The plates were incubated at 37 °C
76 in 5% CO2 incubator for 7 days, before colonies were counted using a dissecting microscope. Titres
77 were determined as 1.63x108 and 4.7x107 CFU/ml for MG and MS respectively.
78
79 **Swabs**
80 The performances of the following types of cotton tip dry swabs without transport medium were
81 compared: wooden shaft and plastic shaft (Alpha Laboratories, Ltd, UK). For each mycoplasma
82 species, each type of swab were used for culture or molecular analysis: swabs were stored at 4 °C
83 and room temperature (RT; 21-23 °C), for 1, 2 and 3 days post inoculation (dpi). At each time
84 point, 8 swabs were sampled of each type. In addition, cotton swabs with plastic shaft in Amies
85 charcoal transport medium (Deltalab, Barcelona, Spain) were used for comparison.
86
87 **Experimental design**
88 MG and MS stock cultures with known titres were serially diluted (neat to 10-7). Each series of
89 wooden or plastic swabs, as well as the charcoal media swabs, were dipped into these broth
90 dilutions for 15 seconds. Subsequently, swabs were stored at either 4 °C or RT as described above.
91 Then, MG and MS recovery was attempted by culture and molecular methods (see below). Both
92 culture recovery and molecular detection were repeated in triplicate for all samples.
93
94 **Mycoplasma recovery by culture**
95 Following storage at different temperatures, each of the dry (plastic and wooden) and charcoal
96 swabs were plated onto MA and incubated at 37 °C in a 5% CO2 incubator. After 7 days of

97 incubation, colonies were quantified using a score from 0 to 4 as previously described (Ley et al.,
98 2003).
99 Molecular detection of mycoplasmas
100 Swabs intended for mycoplasma molecular detection were dipped into 600 µl of working solution
101 D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-
102 mercaptoethanol) (Chomczynski and Sacchi, 2006) and stored at 20 °C for a minimum of three
103 hours. DNA was then extracted using the DNA Mini kit (Qiagen, UK), according to manufacturer's
104 instructions, and stored at 20 °C until use. The extracted DNAs were tested using a duplex PCR
105 targeting the MG mgc2 gene and the MS vlhA gene (Moscoso et al., 2004). DNAs were also tested
106 in duplicate using a commercial quantitative PCR (qPCR) kit for both MG and MS detection
107 (BioChek, Netherlands) on the Rotor-gene Q platform (Qiagen, UK). Obtained Ct values were
108 compared against a previously established standard curve (data not shown) of known
109 concentrations, where relative log REU values were obtained.
110
111 Statistical analysis
112 Detection limits obtained from both culture or conventional PCR were analysed to identify
113 statistically significant differences using Student t-test. A P-value <0.05 was considered statistically
114 significant.
115
116 Results
117
118 Mycoplasma recovery by culture
119 M. gallisepticum: Culture of MG from swabs stored at RT showed that recovery was significantly
120 more efficient for plastic (7.62x102 CFU/ml) than wooden (3.49x105 CFU/ml) swabs (P<0.01)
121 (Figure 1A). Plastic swabs also had the greatest detection ability for MG culture from swabs stored

at 4 °C (3.49x102 CFU/ml) compared to RT (7.62x102 CFU/ml) (Figure 1A), though there were no significant differences. The same was true at 2-3 dpi, as the plastic swabs showed greater detection ability compared to the wooden swabs, with only the high concentration sample (1.17x108 CFU/ml) showing a successful culture from wooden swabs by 3 dpi.

M. synoviae: By 1 dpi we were able to isolate MS to a minimum of 4.7x103 CFU/ml from plastic and wooden swabs stored at 4oC and plastic swabs stored at RT. The ability to re-detect MS from plastic swabs did not alter throughout for either temperature. No MS were isolated from wooden swabs stored at RT at 1 and 3 dpi, however high concentration samples were detected at 2 dpi (Figure 1B).

Molecular detection of mycoplasmas

M. gallisepticum: At 1 dpi, minimum PCR detection limits were on average significantly lower for plastic (3.49x103 CFU/ml) compared to wooden (7.62x104 CFU/ml) swabs when stored at RT ($P<0.05$), whereas both swab types stored at 4 °C showed no difference in detection limits (Figure 1C). At later sampling points, the plastic swabs showed a greater ability to detect MG for both incubation temperatures. Similarly, the MG qPCR assay had greater detection capability when applied to plastic swabs stored at RT (1.63x104 CFU/ml) compared to wooden swabs (1.63x105 CFU/ml) at 1dpi. However, similar to PCR data, both swab types showed the same sensitivity at 4 °C (Figure 1E). At 2 and 3 dpi, only the wooden swabs stored at 4 oC were positive for MG, with all plastic swabs positive, although only at a higher concentration (1.17x108) compared to 1 dpi.

M. synoviae: By PCR, plastic swabs showed a lower minimum detection limit compared to wooden swabs when stored for 24 hours at 4 °C (4.7x105 CFU/ml and 1x106 CFU/ml) and a significantly ($P<0.05$) lower result when stored at RT (1x105 CFU/ml and 2.2x106 CFU/ml respectively) (Figure 1D). At 2-3 dpi, it was only possible to detect MG from the plastic swabs. In contrast, the MS qPCR showed the same detection sensitivity at 1 dpi for both types of swabs at RT (1x104 CFU/ml), but a

147 greater efficiency when applied to plastic swabs at 4°C (plastic = 2.2x103 CFU/ml; wooden =
148 4.7x103 CFU/ml) (Figure 1F). Results at 2 and 3 dpi were similar to the PCR detection, with no
149 wooden swabs positive for MS.
150
151 Discussion
152 Typically, when potentially infected poultry are sampled for mycoplasma detection, cotton tipped
153 swabs are transported to the laboratory by the following day, however this may take several days
154 depending on the location and method. While it is advised that transportation should also include
155 ice or a cold pack to preserve sample integrity, it may not always be possible. For this reason, we
156 investigated the influences of storage at two temperatures (4oC and room temperature), and
157 several incubation times (1-3 dpi) on recovery of MG and MS using molecular and traditional
158 culture methodologies. Previous work has highlighted the difference between swab types
159 (Ferguson-Noel et al., 2012; Zain and Bradbury, 1995); however we report the first study to
160 combine the use of traditional culture analysis (both agar and broth), with modern molecular
161 detection methods.
162
163 Findings from this study showed that dry plastic and charcoal swabs (both with a plastic shaft) had
164 a similar ability to detect MG via culture when stored at 4 oC and RT. In contrast, while not
165 significant, it appears that charcoal swabs were more effective for culturing MS when stored at 4
166 oC, with both plastic shaft swabs out-performing the wooden shaft. For both MG and MS, the dry
167 plastic and charcoal swabs had a greater sensitivity to recover when stored at 4 oC, suggesting that
168 transporting swab samples on ice is advantageous for successful detection (Zain and Bradbury,
169 1996).
170

In this study, the charcoal swabs showed a similar level of detection, irrespective of the storage temperature or duration, perhaps due to the preserving properties of charcoal medium, negating the effects of temperature fluctuations. The type of transport media and swab type used for sample preservation has shown to vary in ability to culture both aerobic and anaerobic bacteria (Tan *et al.,* 2014), with a possible reduction in recovery ability after 24 hours (Roelofsen *et al.,* 1999).

On culture of mycoplasmas, it appears that for both MG and MS, samples collected using wooden swabs and stored at RT could be detrimental for the detection of these organisms, either by isolation or PCR (especially for MS). In this study, although a reduced number of colonies were recovered for MG, no viable colonies were recovered for MS from wooden swabs stored at RT following either 1 or 3 dpi. Similarly, reduced levels of MG or MS detection were found in wooden swabs stored at RT when detection was attempted by PCR. The growth rate and viability of MG and MS can be also affected by the pH of the broth (Lin *et al*., 1983; Ferguson-Noel *et al.*, 2013) and it was previously hypothesized that greater humidity and lower temperature protected against the effect of low pH (Zain and Bradbury, 1996). This could be particularly true for MS, which may no longer be viable under a low pH (Ferguson-Noel *et al.*, 2013). In the present study, while the broth pH was not measured during incubation, a colour indicator alteration suggested an alteration in pH, alongside the difference in physical features of the wooden compared to the plastic swab (Ismail *et al.,* 2013).

Using molecular methods to detect MG, plastic swabs at RT initially displayed the greatest sensitivity. This could be related to permissive mycoplasma growth temperatures, which ranged from 20 to 45°C (Brown *et al.*, 2011). Previous work has reported that MG grown in mycoplasma broth and incubated at room temperature initially shows an increased titre up to 8 hours post

inoculation, followed by a rapid decline in viability (Christensen *et al.*, 1994). Additionally, Zain and Bradbury (1996) demonstrated that the viability of MG on wet swabs reduces following 4 h of incubation at 24-26 °C. In the present study, molecular data showed that while the total genomic presence (viable and non-viable) increased, the number of viable colonies decreased when swabs were stored at RT. This was further emphasised at 2 and 3 dpi, as only the samples containing the highest concentrations of MG and MS were detected from plastic swabs, with no detections possible at RT (MG) or any temperature (MS) from wooden swabs.

In conclusion, results from the current study suggest that swabs with a plastic shaft should be preferred over the wooden shaft for MG and MS detection by culture, PCR and qPCR. While a lower storage temperature (4°C) is better for culture recovery, it seems that both temperatures investigated here are adequate for molecular detection, and the swab type is the bigger factor in determining a positive recovery.

Acknowledgement

The authors would like to thank BioChek for the qPCR kits that were used for this study.

Conflict of Interest

All authors declare that they have no conflict of interest.

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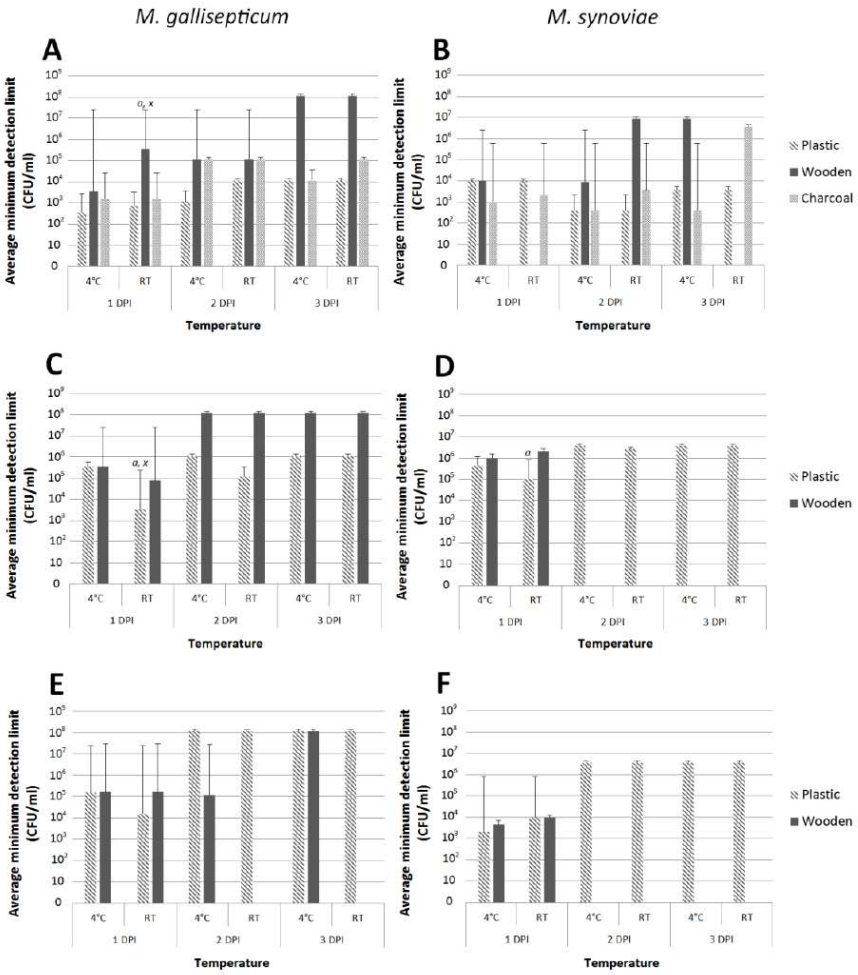
List of figures

Figure 1. Comparison of each swab type following storage at 4 oC and room temperature (RT). (A) Culture efficiency for MG; (B) Culture efficiency for MS; (C) PCR detection of MG; (D) PCR detection of MS; (E) qPCR detection of MG; (F) qPCR detection of MS. Data shown as mean of the highest dilution producing a positive culture result, with standard error margins. Groups with the notation of 'a' indicate significant (P<0.05) differences within the same temperature, whereas 'x' indicates significant differences against the corresponding group at the different temperature.



Comparison of each swab type following storage at 4 oC and room temperature (RT). (A) Culture efficiency for MG; (B) Culture efficiency for MS; (C) PCR detection of MG; (D) PCR detection of MS; (E) qPCR detection of MG; (F) qPCR detection of MS. Data shown as mean of the highest dilution producing a positive culture result, with standard error margins. Groups with the notation of 'a' indicate significant ($P<0.05$) differences within the same temperature, whereas 'x' indicates significant differences against the corresponding group at the different temperature.